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The dynamics of calcium oscillations that activate mammalian eggs

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ABSTRACT It has been known for some time that mammalian eggs are activated by a series of intracellular calcium oscillations that occur shortly after sperm egg membrane fusion. Recent work has identified a novel sperm specific phospholipase C zeta as the likely agent that stimulates the calcium oscillations in eggs after sperm-egg membrane fusion. PLCzeta is stimulated by low intracellular calcium levels in a manner which suggests that there is a regenerative feedback of calcium release and PLCzeta induced inositol 1,4,5-trisphophate ($InsP_3$) production in eggs. This implies calcium oscillations in fertilizing mammalian eggs are driven by underlying oscillations of $InsP_3$. This model of oscillations is supported by the response of mouse eggs to sudden increases in $InsP_3$. The cellular targets of calcium oscillations include calmodulin-dependent protein kinases, protein kinase C and mitochondria. There is evidence that eggs might be best activated by multiple calcium increases rather than a single calcium rise. As yet we do not fully understand how the target of calcium in a mammalian egg might decode the patterns of calcium changes that can occur during egg activation.

KEY WORDS: *fertilization, egg, sperm, calcium, oscillations*

Introduction

At fertilization the egg is activated to begin development. Egg activation can involve a multitude of cellular changes depending upon the species. The most widely observed changes in mammals are the completion of meiosis, the exocytosis of cortical granules, changes in the pattern of protein synthesis and the formation of pronuclei indicating the start of the first zygotic cell cycle (Ducibella *et al.,* 2006; Stricker, 1999; Runft *et al.,* 2002; Whitaker 2006). A role for Ca^{2+} in directly causing the events egg activation at fertilization was first indicated when it was reported that application of this Ca^{2+} ionophore, to causes a rise in cytosolic Ca^{2+}, was able to induce the early events of activation in eggs of a wide range of species including those from hamsters (Steinhardt *et al.,* 1974). Subsequently, it was also shown that microinjection of Ca^{2+} ions alone could trigger embryo development up to the blastocyst stage in the mouse (Fulton and Whittingham, 1978). The importance of Ca^{2+} changes was then later demonstrated by showing that chelation of Ca^{2+} ions using BAPTA could block all signs of activation in mouse eggs (Kline and Kline, 1992a). These data suggest that a Ca^{2+} increase in the egg is a universal trigger for the activation of development in mammals. The first measurements of Ca^{2+} at fertilization were in medaka fish eggs, and then sea urchin eggs, where the Ca^{2+} sensitive photoprotein aequorin was used to show that a wave of cytosolic free Ca^{2+} increase crosses the egg after sperm interaction (Ridgway *et al.,* 1977; Steinhardt *et al.,* 1977). These studies suggested that a single Ca^{2+} increase that lasts several minutes is the cause of egg activation in these species.

In 1981 two papers were published that suggested that the free Ca^{2+} changes in mammalian eggs might be more complex than in fish or sea urchin eggs. Firstly measurements of the membrane potential in fertilizing hamster eggs showed a series of repetitive hyperpolarizations within 10 seconds of sperm egg interaction (Igusa and Miyazaki 1981). Since the hyperpolarizations appeared to be due to a Ca^{2+}-activated K^+ conductance the data suggested that hamster eggs underwent period Ca^{2+} changes at fertilization (Igusa and Miyazaki, 1982). Other studies using aequorin to measure Ca^{2+} directly also suggested that fertilization in mouse eggs was accompanied by periodic increases in cytosolic free Ca^{2+} (Cuthberston *et al.,* 1981; Cuthbertson and Cobbold, 1985). These data in mouse and hamster eggs were backed up

Abbreviations used in this paper: $InsP_3$, inositol triphosphate; PLC, phospholipase C; PLCζ, PLC zeta.

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by further studies which confirmed that a series of repetitive Ca^{2+} oscillations occurred for several hours following sperm egg interaction. Studies in many other mammalian species using fluorescent dyes to measure Ca^{2+} have subsequently confirmed that a prolonged series of Ca^{2+} oscillations with a wavelike onset is a characteristic feature of mammalian egg fertilization (Miyazaki *et al.,* 1993). Fig. 1A shows an example of a mouse egg undergoing Ca^{2+} oscillations at fertilization.

We now know that Ca^{2+} oscillations are not unique to mammalian eggs since fertilizing ascidian oocytes also demonstrate a series of Ca^{2+} oscillations at fertilization (Dumollard *et al.,* 2004a). However, the response in mammalian eggs is distinctive in that the oscillations lasts for several hours and involve relatively low frequency, large amplitude Ca^{2+} increases. The mechanism and consequences of these Ca^{2+} oscillations is pivotal for our understanding of how a sperm activates development. This review will focus on the dynamic aspects of Ca^{2+} signalling and discuss how oscillations are generated, and how they may be transformed into a response by the egg. Some of the potential downstream targets of Ca^{2+} signals in eggs, such as myosin light chain kinase, or src family protein kinases (Matson *et al.,* 2006; McGinnis *et al.,* 2007), are not discussed since there is no explicit dynamic data on their activity at fertilization.

Ca^{2+} release at fertilization in mammalian eggs is generated by $InsP_3$ production

The Ca^{2+} oscillations in mammalian eggs appear to be a result of Ca^{2+} release via the $InsP_3$ receptor (predominantly type 1) that is both the receptor for $InsP_3$ and a Ca^{2+} release channel (Kurakawa *et al.,* 2004). Studies in mouse and hamster eggs demonstrated that blocking the $InsP_3$ receptor with a specific antibody inhibits Ca^{2+} oscillations at fertilization (Miyazaki *et al.,* 1993). Furthermore, injecting adenophostin to cause the down-regulation of $InsP_3$ receptors before fertilization, can block all Ca^{2+} oscillations in subsequently fertilized eggs (Brind *et al.,* 2000; Jellerette *et al.,* 2000). Reducing the expression of $InsP_3$ receptors by injection of siRNA can also inhibit subsequent Ca^{2+} oscillations in fertilizing mouse eggs (Xu *et al.,* 2003). Other Ca^{2+} releasing agents such as cyclic ADP ribose, nitric oxide or NAADP do not appear to cause Ca^{2+} release in mouse eggs and so other Ca^{2+} release channels are probably not directly involved in generating Ca^{2+} oscillations. These data all show that that the $InsP_3$ receptor is the essential messenger for Ca^{2+} release in mammalian eggs fertilization.

The essential role of the $InsP_3$ receptor implies that increased $InsP_3$ production occurs at fertilization in eggs. Increases in phosphoinositide turnover and $InsP_3$ production have been demonstrated from biochemical and radiolabelling assays in sea urchin and frog eggs (Whitaker, 2006). However, this is impractical in mammalian eggs because thousands of eggs are required. However, a useful probe for measuring $InsP_3$ production in single cells is GFP (green fluorescent protein) tagged to the PH (pleckstrin homology) domain of PLCδ1 (Hirose *et al.,* 1999). The translocation of this probe away from the phosphatidyl-inositol bisphosphate (PIP_2) in plasma membrane into the cytoplasm can be caused by increase $InsP_3$ production and so this translocation can be used to indicate $InsP_3$ production (Hirose *et al.,* 1999). When introduced into mouse eggs the GFP-PH domain this probe undergoes an *increase* in plasma membrane labelling with each of the early Ca^{2+} increases (Halet *et al.,* 2002). This is paradoxical since, if fertilization triggers an increase in $InsP_3$ production, the probe would be expected to detach from the plasma membrane and translocate to the cytosol. In fact the data suggest that the probe does not respond to $InsP_3$ changes in mouse eggs, but instead this probe is relocating due to an increase in PIP_2 in the plasma membrane (Halet *et al.,* 2002).

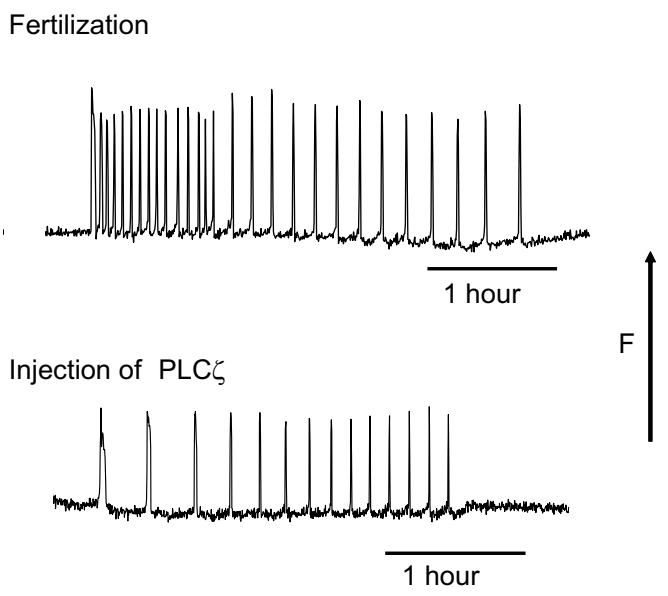

Fig. 1. Intracellular Ca^{2+} oscillations in mouse eggs measured by the fluorescence of a Ca^{2+} sensitive dye (Oregon green BAPTA dextran). *See Campbell and Swann (2006) for experimental details. In* **(A)***, an egg is shown in response to fertilization, and in* **(B)***, the response of an egg is shown after microinjection of cRNA for mouse PLCζ (Campbell and Swann, 2006; Saunders* et al.*, 2002). The fluorescence F on the y-axis is in arbitrary units, so the Ca^{2+} levels are not calibrated.*

Although it has proved difficult to use fluorescent $InsP_3$ indicators in fertilizing mouse eggs (but see later), sperm induced $InsP_3$ production in mammalian eggs can be inferred from the finding that fertilization leads to a marked down-regulation in the number of $InsP_3$ receptors (Brind *et al.,* 2000; Jellerette *et al.,* 2000). This down-regulation is not due to the Ca^{2+} increase and can only be induced by agents that bind to the $InsP_3$ receptor on the $InsP_3$ binding site. This data clearly suggest that $InsP_3$ does increase at fertilization in mammalian eggs. The key question is how the sperm produces the increase in $InsP_3$ levels.

Signalling by a sperm factor

There have been many different ideas for how sperm may cause $InsP_3$ production and Ca^{2+} release in eggs. These are covered in many previous reviews (Stricker 1999, Kurakawa *et al.,* 2004; Runft *et al.,* 2002; Swann *et al.,* 2006; Whitaker, 2007). A consensus of opinion has emerged that Ca^{2+} signalling in mammalian fertilization is initiated by the introduction of a sperm factor into the egg after sperm-egg membrane fusion. This fusion event occurs a few minutes before the first Ca^{2+} release in the mouse egg and this means there is ample time for a factor to

diffuse into the egg (Lawrence *et al.,* 1997; Jones *et al.,* 1998a). The initial direct evidence for the existence of such a soluble sperm factor came from the observation that injecting soluble sperm extracts could trigger Ca^{2+} oscillations similar to those seen at fertilization in hamster and mouse eggs (Swann, 1990). Such a sperm factor has now been shown in other species such as pigs and humans (Kurakawa *et al.,* 2004; Swann *et al.,* 2006). Important clues to the nature of the sperm factor came from studies using the sea urchin egg homogenate. It was found that the mammalian sperm factor causes a large increase in $InsP_3$ and Ca^{2+} release from intracellular vesicles in this egg homogenate (Jones *et al.,* 1998b). Similarly injection of the sperm factor into frog eggs has been shown to cause and increase in $InsP_3$ that can be measured by bioassay (Wu *et al.,* 2001). Injecting the sperm protein factor into mouse eggs has also been shown to cause down regulation of $InsP_3$ receptors which is a sign that an $InsP_3$ increase has occurred (Lee *et al.,* 2006). The work in sea urchin egg homogenates also showed that the sperm extract themselves possessed a high PLC activity (Jones *et al.,* 1998b; Rice *et al.,* 2000). This suggested that the sperm factor was itself a phosphoinositide (PI) specific PLC. The problem with this hypothesis was that injecting the known isoforms of PI specific PLC did not cause Ca^{2+} oscillations in eggs (Jones *et al.,* 2000), or else where they were reported to cause Ca^{2+} oscillations, such as with PLCγ, the concentrations of PLC were hundreds of times greater than the PLC activity found in a single sperm (Mehlmann *et al.,* 2001). The resolution to this conundrum came with discovery of a novel, sperm specific, PLC isoform.

PLCζ and Ca^{2+} release at fertilization

PLC zeta (PLCζ) was first identified as a novel form of PLC expressed exclusively in testis (Saunders *et al.,* 2002). It has a molecular mass of ~70kDa which makes it the smallest of the mammalian PI specific PLCs (Rebecchi and Pentyala 2000). It is closest in primary sequence to the δ class of PLCs. PLCζ consist of four EF hand domains that bind Ca^{2+}, a C2 domain, and X and Y catalytic domains found in all mammalian PLCs. It is notably different from most other mammalian phosphoinositide specific PLCs in lacking a PH domain. When the cRNA for PLCζ or recombinant PLCζ protein is injected into mouse eggs it triggers Ca^{2+} oscillations very similar to those seen at fertilization (Saunders *et al.,* 2002; Kouchi *et al.,* 2004). Fig. 1B shows an example of a mouse egg undergoing Ca^{2+} oscillations after injection of PLCζ cRNA. Immunodepletion of PLCζ from sperm extracts abolishes their ability to cause Ca^{2+} oscillations in mouse eggs. This suggests that the previously described sperm factor is PLCζ (Swann *et al.,* 2006; Saunders *et al.,* 2007).

There is every indication that PLCζ is involved in causing Ca^{2+} oscillations in fertilizing mammalian eggs. A PLCζ isoform has now been identified in at eight mammalian species and in many cases it has been shown to trigger Ca^{2+} oscillations in eggs (Swann *et al.,* 2006). The amount of PLCζ that is required to trigger Ca^{2+} oscillations is within the range of PLCζ in a single sperm (Saunders *et al.,* 2002). The release of PLCζ from sperm probably occurs well within the first hour after sperm-egg fusion and this correlates with the time over which the Ca^{2+} oscillations occur (Yoon and Fissore, 2007). There also now evidence that PLCζ is the factor responsible for causing Ca^{2+} oscillation after intracytoplasmic sperm injection (Fujimoto *et al.,* 2004).

Only one study to date has directly addressed the requirement of PLCζ in normal fertilization. The knockdown of PLCζ in sperm using a transgenic RNAi approach has been shown to reduce the number of Ca^{2+} oscillations and reduce the activation rates at fertilization (Knott *et al.,* 2005). This transgenic approach generates mosaic expression in spermatogenic cells, and some sperm would still contain some PLCζ that could account for why some sperm can still cause some Ca^{2+} oscillations and egg activation. One noteworthy feature, however, is that the transgene was never passed on from males to the next generation. This implies that sperm carrying the transgene, where PLCζ would be most reduced, are not able to trigger an egg to activate and develop. This implies that PLCζ is required for normal fertility in male mice (Knott *et al.,* 2005). The actions of PLCζ appears to be specific to egg since transgenic expression of PLCζ in somatic cells appears to have little effect. In the ovary PLCζ expression it leads to spontaneous egg activation and the subsequent development of ovarian teratocarcinomas (Yoshida *et al.,* 2007).

The mechanism of action and localization of PLCζ in eggs is unclear. Studies using a Venus-tagged (hence highly fluorescent) version of PLCζ have suggested that it is not specifically localized in the plasma membrane (Yoda *et al.,* 2004). The apparent lack of specific plasma membrane localization could be due to the fact that PLCζ lacks a PH domain which localizes the closely related PLCδ1 to the PIP_2 in plasma membrane (Saunders *et al.,* 2002). The parts of PLCζ that may be involved in localization to a source of PIP_2 are the C2 domain and a region between the X and Y catalytic domains called the X-Y linker (Kouchi et val. 2005; Nomikos *et al.,* 2005). The X-Y linker region has several basic residues that could help anchor it to PIP_2 which is a very negatively charged phospholipid (Nomikos *et al.,* 2007). However, it is still unclear whether the PIP_2 that PLCζ binds to is in the plasma membrane or an internal organelle. The only statement we can make about its localization is that PLCζ enters the pronuclei as they form after mouse egg activation (Larman *et al.,* 2004; Yoda *et al.,* 2004;Yoon and Fissore, 2007). This localization appears to involve a nuclear targeting region in the X-Y linker region of the protein that is the same as that proposed to bind to PIP_2. The localization of PLCζ in pronuclei is associated with the termination of Ca^{2+} oscillations and may act in conjunction with modifications to the $InsP_3$ receptor to terminate Ca^{2+} signals at fertilization (Larman *et al.,* 2004; Lee *et al.,* 2006). More extensive reviews of PLCζ can be found elsewhere (Swann *et al.,* 2006; Saunders *et al.,* 2007).

How does Ca^{2+} oscillate in an egg?

The discovery of PLCζ provides a foundation for understanding how Ca^{2+} oscillations are generated at fertilization. One of the features that distinguishes PLCζ from other mammalian PLCs is the extraordinary sensitivity to Ca^{2+} ions due to the presence of EF domains that bind Ca^{2+} (Kouchi *et al.,* 2005; Nomikos *et al.,* 2005). The high sensitivity to Ca^{2+} was observed previously for the sperm factor associated PLC activity (Rice *et al.,* 2000). Most, if not all, phosphoinositide specific PLCs show some stimulation by Ca^{2+} ions, but the level of Ca^{2+} required to stimulate the isolated enzyme is in the micromolar range which is well above the resting levels in cells (Rebecchi and Pentyala 2000). In contrast recom-

binant PLCζ is stimulated by Ca^{2+} in the 100nM-800nM range *in vitro*. This means that once PLCζ is in the egg it is expected to generate $InsP_3$ at resting Ca^{2+} concentrations in eggs (~100nM). Furthermore, when Ca^{2+} levels start to increase there will be a further increase in $InsP_3$ production that will enhance the size of the Ca^{2+} increase. It is possible that egg-derived PLCs, such as PLCβ, could also contribute to $InsP_3$ generation during the peak of such Ca^{2+} rises (Igirashi *et al.,* 2007). This all implies a positive feedback of increased Ca^{2+} and $InsP_3$ during the rising phase of each Ca^{2+} increase. Such positive feedback plays a central role in models of Ca^{2+} oscillations in some somatic cells (Meyer and Stryer, 1988; Harootunian *et al.,* 1991; Hirose *et al.,* 1999; Politi *et al.,* 2006). The only explicit mathematical model of Ca^{2+} signals in eggs at fertilization to date is in ascidians where the best way of explaining repetitive Ca^{2+} waves is to assume that a highly Ca^{2+} sensitive PLC generated $InsP_3$ induced Ca^{2+} release (Dupont and Dumollard, 2004). Such an idea of Ca^{2+} dependent $InsP_3$ production in eggs dates back to one proposed to account to the singular Ca^{2+} wave at fertilization in the sea urchin egg (Whitaker and Irvine, 1983). It is possible that this idea could also account for waves and oscillations in mammalian eggs.

Although regenerative $InsP_3$ production is a plausible mechanism in eggs exact mechanism of Ca^{2+} oscillations has remained unresolved because there is another popular model of Ca^{2+} oscillations that involves positive and negative feedback loops of Ca^{2+} acting directly on the $InsP_3$ receptor (De Young and Keizer, 1992). In this model at low Ca^{2+} levels the $InsP_3$ receptor is stimulated by Ca^{2+}, but at higher Ca^{2+} levels the $InsP_3$ receptor is closed by Ca^{2+} (Bezprozvanny *et al.,* 1991). If this model is correct the $InsP_3$ does not oscillate but instead it provides constant stimulation to the $InsP_3$ receptor that undergoes oscillatory openings. This idea has also received experimental verification in somatic cells. A fluorescent resonance energy transfer (FRET) indicator called IRIS-1, based upon the $InsP_3$-binding domain of the $InsP_3$ receptor, has been used to show that $InsP_3$ does not oscillate during metabotropic glutamate receptor stimulation of Ca^{2+} oscillations in HeLa cells (Matsu-ura *et al.,* 2006). This model is supported in eggs by the finding that sustained injection of $InsP_3$ or its non hydrolysable derivative, or adenosphostin, or generating a sustained small $InsP_3$ increase by photorelease, can all lead to a series of Ca^{2+} oscillations in unfertilized hamster or mouse eggs (Swann *et al.,* 1989; Swann, 1994; Galione *et al.,* 1994; Nixon and Jones, 2000; Jellerette *et al.,* 2000). This suggests that the $InsP_3$ receptor alone can generate oscillations and that $InsP_3$ levels do not need to oscillate.

One way to try and resolve the mechanism of Ca^{2+} oscillations is to measure $InsP_3$ during fertilization. As, noted above, the GFP-PH indicator used in many somatic cells is not useful for measuring $InsP_3$ in mammalian eggs. However, a different FRET based fluorescent indicator called fretino has been used to study the dynamics of $InsP_3$ in mouse eggs (Shirakawa *et al.,* 2006). Like IRIS, this indicator is based upon $InsP_3$ binding to the binding-domain of the $InsP_3$ receptor which contains two attached fluorescent proteins (Shirakawa *et al.,* 2006). At fertilization this probe suggested there is an increase $InsP_3$, but the signal change is very small and not obviously oscillatory. However, after PLCζ cRNA injection the Ca^{2+} oscillations eventually become associated with distinct oscillations in $InsP_3$ (Shirakawa *et al.,* 2006). One interpretation of these data is that the indicator is not sensitive enough to measure $InsP_3$ oscillations at fertilization but when PLCζ is introduced into the egg via RNA injection there is a gradual increase in PLCζ levels to that somewhat above physiological. This high level of PLCζ is then sufficient to generate enough $InsP_3$ to be measurable as oscillations. However, it is also possible to argue that physiological levels of PLCζ at fertilization are insufficient to lead to the $InsP_3$ oscillations, or that $InsP_3$ oscillations are an epiphenomena and not central to the oscillatory mechanism.

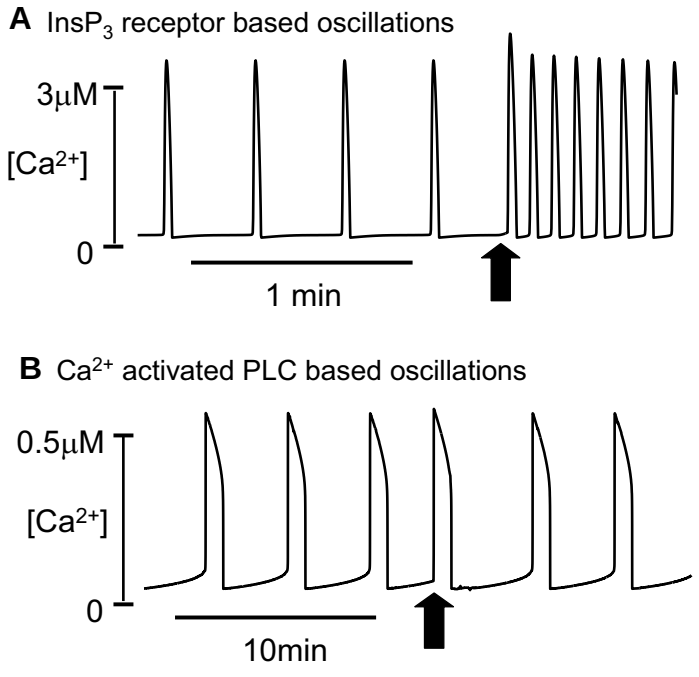

Fig. 2. Different models of Ca^{2+} oscillations respond differently to pulses of $InsP_3$. *In* **(A)***, a simulation is shown for a model in which positive and negative feedback on the $InsP_3$ receptor generate the oscillations. A sudden increase in $InsP_3$ (indicated by the arrow) causes an increase in the frequency of oscillations. For the sake of illustration, in this case, $InsP_3$ is slowly metabolized and so the increase lasts for some time, but a pulse of $InsP_3$ in this model leads to an increase in the frequency of oscillations (Sneyd* et al.*, 2006). In* **(B)***, a simulation is shown for oscillations generated by Ca^{2+} induced feedback on $InsP_3$ production. A sudden increase in $InsP_3$ leads to a Ca^{2+} transient that resets the oscillatio cycle, with no increase in frequency. The model and parameters are described in Sneyd* et al. *(2006).*

Distinguishing between different models of Ca^{2+} oscillations

It is difficult to distinguish between the two different models for Ca^{2+} oscillations. Either one can apply depending upon the cell type and stimulus. One method proposed to distinguish between mechanisms is based upon introducing a sudden increase in $InsP_3$ using, for example, photo-release of a caged $InsP_3$ (Sneyd *et al.,* 2006). The effect of a sudden pulse of $InsP_3$ has very different effects depending upon the model of Ca^{2+} oscillations. If the model has $InsP_3$ as a control parameter ($InsP_3$ receptor based oscillations) then any transient increase in $InsP_3$ leads to an increase in the frequency of Ca^{2+} oscillations. If, however, $InsP_3$

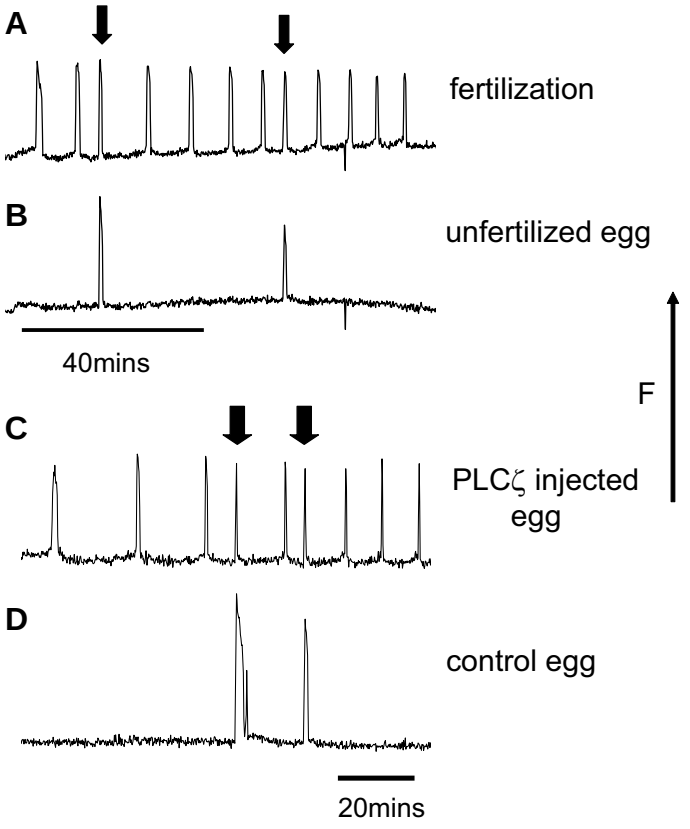

Fig. 3. Photorelease of caged $InsP_3$ in mouse eggs. *All eggs were injected with caged $InsP_3$ (0.5 mM in pipette) and Oregon green BAPTA dextran and conditions are as in Fig. 1. In* **(A)**, *a fertilizing egg was exposed to (5s) pulses of UV light from a mercury lamp (with a UG11 filter) at the times indicated by the arrows. In* **(B)**, *an egg that was in the same dish but that had not fertilized was exposed to the same UV pulses at the same time. In* **(C)**, *an egg injected with PLCζ cRNA was exposed to a (10s) UV pulse during oscillations, and* **(D)** *shows an uninjected egg in the same dish that also responded to the UV pulses. In all cases a large Ca^{2+} rise followed the photo-release of $InsP_3$. In eggs undergoing oscillations, there was no obvious increase in the frequency of Ca^{2+} oscillations either at fertilization (example from 5 eggs), or after PLCζ injection (example from 12 eggs).*

is a dynamic component of the oscillation mechanism (regenerative $InsP_3$ production) then a sudden pulse increase in $InsP_3$ will cause a Ca^{2+} transient that resets the oscillation cycle and does not increase the frequency Ca^{2+} transients. This difference in response to perturbation is entirely robust with respect to other details of the mathematical model and it has been confirmed with 13 different models of Ca^{2+} oscillations (Sneyd *et al.,* 2006). It provides a simple and qualitative way of discriminating which model of oscillations is operative in a cell.

In Fig. 2 some example simulations are shown for the two different types of mathematical model of Ca^{2+} oscillations (for details see Sneyd *et al.,* 2006). In the case where oscillations occur via $InsP_3$ receptor feedback the sudden increase in $InsP_3$ leads to a marked increase in frequency of Ca^{2+} oscillations (Fig. 2A). In the case where $InsP_3$ undergoes regenerative increases, the sudden increase in $InsP_3$ causes a large Ca^{2+} increase but this does no lead to extra oscillations, but instead resets the oscillation cycle (Fig. 2B). Fig. 3 shows examples of experiments where $InsP_3$ was increased suddenly in a fertilizing, or PLCζ injected, mouse eggs by photo-release of caged $InsP_3$. In each case there is a sudden rise of Ca^{2+} that is similar to that seen in control unfertilized eggs. However, this does not lead to any sign of extra Ca^{2+} oscillations. Instead it resets the period of Ca^{2+} oscillations and the next transient occurs with a delay similar to the period prior to the pulse of $InsP_3$. These results are consistent with the model of oscillations in which Ca^{2+} induced $InsP_3$ production is involved in each Ca^{2+} rise (Hirose *et al.,* 2002).

To reconcile the data in Fig. 3 with previous data on $InsP_3$ induced Ca^{2+} oscillations, we suggest that two mechanisms of Ca^{2+} oscillations exist in mammalian eggs in a manner illustrated in Fig. 4. In an unfertilized mammalian egg the $InsP_3$ receptor alone provides the mechanism for generating Ca^{2+} oscillations that are seen, for example, in response to sustained $InsP_3$ increases (Swann *et al.,* 1989; Swann 1994; Nixon and Jones, 2000). This mechanism could also explain the spontaneous Ca^{2+} oscillations that are seen in immature mouse oocytes shortly after isolation from the ovary (Carroll and Swann, 1992; Nixon and Jones, 2000). After fertilization the sperm will have introduced PLCζ and as a result oscillations are generated by a different mechanism involving a regenerative rise in $InsP_3$ during each Ca^{2+} transient. The regenerative and Ca^{2+} dependent $InsP_3$ production in Fig. 4B also offers an explanation for the enhanced sensitivity of the eggs to 'Ca^{2+} induced Ca^{2+} release' (Igusa and Miyazaki, 1983). The so called 'Ca^{2+} induced Ca^{2+} release' could actually be Ca^{2+} induced $InsP_3$ production which is only seen after fertilization because of the presence of PLCζ (Shirakawa *et al.,* 2006)

In support of the mechanisms proposed in Fig. 4 it is noteworthy that models involving just the kinetics of the $InsP_3$ receptor tend to produce high frequency short duration oscillations with the time periods of less than a minute (Politi *et al.,* 2006). This is why the mathematical model of Ca^{2+} oscillations based upon the $InsP_3$ receptor in Fig. 2 are rather high frequency. This relatively high frequency response is similar to what is observed in immature oocytes, and in unfertilized hamster and mouse eggs injected with agents that stimulate the $InsP_3$ receptor (Carroll and Swann, 1992; Swann *et al.,* 1989; Galione *et al.,* 1994; Swann, 1994; Jones and Nixon, 2000; Jellerette *et al.,* 2000). In contrast models involving positive feedback of Ca^{2+} on $InsP_3$ production can produce a much greater range of Ca^{2+} oscillations including those with time periods of many minutes as seen with fertilizing eggs (Politi *et al.,* 2006). There could also be Ca^{2+} dependent metabolism of $InsP_3$ which would mean that $InsP_3$ oscillations would be even more strongly coupled to Ca^{2+} oscillations (Politi *et al.,* 2006).

Although most of the debate over different ideas for oscillations revolves around the rising phase of each Ca^{2+} transient, there are various Ca^{2+} pumps such as the SERCA pumps that play an important role in returning Ca^{2+} to the Ca^{2+} stores (Kline and Kline, 1992b). There is also some Ca^{2+} flux out of the cell due to a plasma membrane Ca^{2+} ATPase, and a Na^+/Ca^{2+} exchanger (Georgou *et al.,* 1998; Carroll 2000). In compensation for the efflux, a small influx of Ca^{2+} into the egg appears to occur during each Ca^{2+} transient (McGuinness *et al.,* 1996). Ca^{2+} uptake into mitochondria occurs (Dumollard *et al.,* 2007), and it is possible that a cycle of Ca^{2+} through other compartment, such as the mitochondria,

could play a role in setting up the frequency of Ca^{2+} oscillations (Ishii *et al.,* 2006). However, the Ca^{2+} dynamics inside the various compartments in eggs has not yet been reported.

How might an egg decode Ca^{2+} oscillations?

The multiple increases in Ca^{2+} trigger all the major events at fertilization. Furthermore, with each Ca^{2+} increase there is a progressive stimulation of each process (Ducibella *et al.,* 2006). This is shown most directly by using repetitive electroporation to drive different numbers of Ca^{2+} increases in eggs and induce parthenogenetic activation (Ozil and Hunaeu 2001). Some events such as meiotic resumption are stimulated early by a single Ca^{2+} transient, whilst other events such as the decreases in activity of cell cycle protein kinases requires more Ca^{2+} transients (Ducibella *et al.,* 2002). Exocytosis occurs in a stepwise manner with each pulse of Ca^{2+} stimulating a loss of cortical granules from the egg (Ducibella *et al.,* 2002). This suggests that, during normal fertilization, mammalian eggs respond in an integral way to pulses of Ca^{2+} release. In turn this implies that some downstream components in the egg may read out the number, or frequency, of Ca^{2+} increases in eggs (Dupont and Goldbeter, 1998). It is of particular interest, therefore, to examine enzymes or signalling modules that can be seen to be 'decoding' the oscillatory signal.

One of the immediate targets of the Ca^{2+} increases in eggs is the cell cycle machinery that keeps the egg arrested in metaphase of the second meiosis. The resumption of meiosis is stimulated through a series of changes in the activity of protein kinases (Carroll, 2001; Jones 2007). This results in the stimulation of the anaphase promoting complex/cyclosome which leads to proteolysis of cyclin B that is required to maintain the CDK1 (cell division cycle kinase) activity responsible for arrest in meiosis II (Carroll 2001; Jones 2007). The dynamics of cyclin B destruction has been measured at fertilization using GFP-tagged cyclin B and it has been shown that cyclin B is rapidly destroyed at the onset of Ca^{2+} oscillations (Nixon *et al.,* 2002). The early studies suggested that each Ca^{2+} spike might lead to a distinct increment in cyclin B destruction (Nixon *et al.,* 2002). However, subsequent studies suggest a non oscillatory, steady decline in cyclin B following the onset of Ca^{2+} oscillations (Marangos and Carroll, 2004). The kinetics of CDK1 activity during fertilization have yet to be determined with the time resolution applied to cyclin B.

Most recent attention has focussed on calmodulin dependent protein kinase II, or CaMKII, as a transducer of the Ca^{2+} signal at fertilization. CaMKII activity is stimulated at fertilization (Markoulaki *et al.,* 2004), and this is significant because it can phosphorylate Emi2 which targets it for destruction and sets in train the activation of the anaphase promoting complex (Jones 2007). The destruction of Emi2 has also been measured with a fluorescent protein tag and its destruction during Sr^{2+} induced egg activation precedes cyclin B destruction, but it is not clear if the destruction is oscillatory (Madgwick *et al.,* 2006). The full effects of CaMKII in eggs have been directly assessed by injection a constitutively activate form of CaMKII (CA-CaMKII). The injection of cRNA for CA-CaMKII into mouse eggs leads to meiotic resumption and initiation of the first cell cycles and development up to the blastocyst stage (Madgwick *et al.,* 2005; Knott *et al.,* 2006). These data suggest that CaMKII is the major transducer of the Ca^{2+} oscillations in fertilizing mammalian eggs. This is of particular

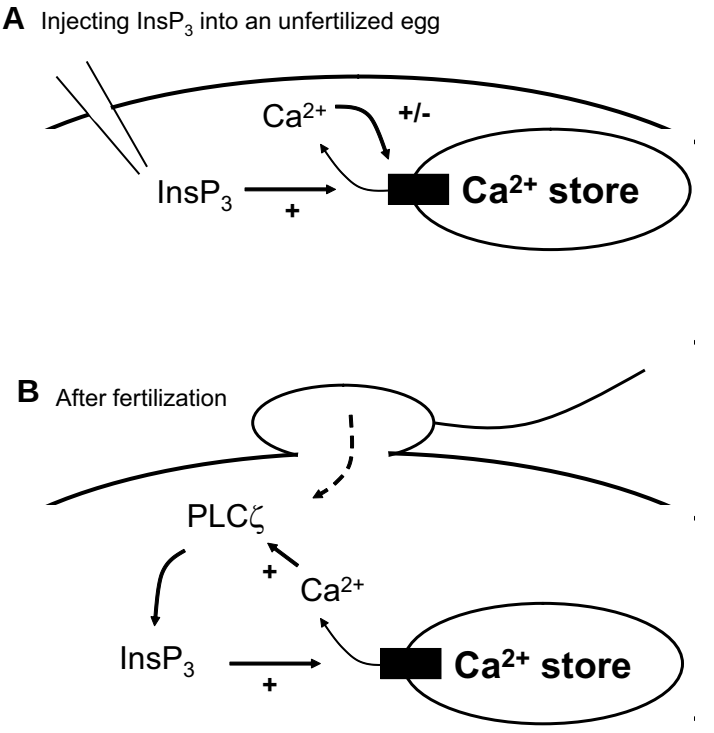

Fig. 4. A schematic diagram to illustrate the different oscillates that are proposed to exist in mammalian eggs. *In both cases, Ca^{2+} release occurs from an intracellular store via the $InsP_3$ receptors (the black rectangle).* **(A)** *When $InsP_3$, or its derivatives, are microinjected into an unfertilized eggs the oscillations are proposed to be solely due to the feedback properties of the $InsP_3$ receptor.* **(B)** *In contrast, after fertilization the introduction of PLCζ introduced a new positive feedback loop which takes over as the mechanism of oscillations.*

interest because CaMKII can undergo auto-activation and stay active after a decrease in cytosolic Ca^{2+} levels (De Konick and Schulman 1998). It can show a level of activity that is a 'read out' of the frequency of Ca^{2+} oscillations (De Konick and Schulman, 1998; Dupont and Goldbeter,1998). However, the data that shows that CaMKII activity acts as such an analogue transducer of Ca^{2+} oscillations in neurons, for example, involves very high frequency Ca^{2+} pulses (>1 Hz) compared with those in mammalian eggs (<0.005 Hz) (De Konick and Schulman, 1998; Dupont and Goldbeter,1998). When the auto-activation of CaMKII is measured in mouse eggs, it undergoes an increase, and then a decrease in activity, essentially in phase with each Ca^{2+} transient (Markoulaki *et al.,* 2004). In other words CaMKII activity does not show any long term memory of each Ca^{2+} pulse. So CaMKII itself, in eggs, does not appear to act as the 'analogue output' of Ca^{2+} oscillations in the way suggested in neurons (De Konick and Schulman, 1998). If there is a decoder of Ca^{2+} oscillations in eggs it probably involves downstream protein substrates of CaMKII that could be reversibly phosphorylated (Dupont and Goldbeter, 1998; Ducibella *et al.,* 2006).

As well as CaMKII, other protein kinases may be involved in turning the Ca^{2+} signals into cellular responses. For example a number of different protein kinase C isoforms are expressed in mammalian eggs, including both the conventional isoforms that can be activated by the Ca^{2+} and diacylglycerol (Halet 2004).

PLCζ is expected to produce DAG as well as $InsP_3$, so this could act in concert to stimulate PKC. Indeed at fertilization there is an increase in PKC activity (Tatone *et al.,* 2003), and different isoforms undergo translocation to either the plasma membrane or meiotic spindle (Tatone *et al.,* 2003; Page Baluch *et al.,* 2004). The stimulation of PKC could play a number of roles at fertilization since phorbol esters, which can mimic the effects of DAG stimulation in stimulating PKC, has been reported to cause egg activation and even trigger small Ca^{2+} oscillations (Cuthbertson and Cobbold, 1985; Colonna *et al.,* 1989). However, the injection of a constitutively activated form of PKCα does not cause Ca^{2+} oscillations or meiotic resumption in mouse eggs so its role in activation is unclear (Madgwick *et al.,* 2004). The role of PKC is made more complex to understand by the existence of non-conventional PKC isoforms that are not stimulated by Ca^{2+}, and the finding that the increase in PKC activity in eggs is not much affected by inhibiting Ca^{2+} transients at fertilization (Tatone *et al.,* 2003; Halet 2004). On the other hand PKC stimulation has the potential to alter Ca^{2+} oscillations at fertilization since either phorbol esters, or a constitutively activate PKC, can enhance Ca^{2+} influx into mouse eggs (Halet *et al.,* 2004; Madgwick *et al.,* 2005).

Of the different isoforms PKCγ is of particular interest with respect to Ca^{2+} oscillations. Rather like CamKII it has been suggested to act a 'decoding machine' for the frequency of Ca^{2+} oscillations (Oancea and Meyer, 1998). The only studies that have addressed the dynamics of Ca^{2+} oscillations and PKC activation have used plasma membrane translocation of GFP linked to either the whole of PKCγ or PKCα, or GFP linked to the C2 or C1 domains of PKCγ (Halet *et al.,* 2004). In many cellular systems the translocation of PKC via its specific domains is linked to enzyme activation so translocation is a surrogate assay for PKC stimulation. These studies have shown that PKC translocation occurs with each Ca^{2+} spike, with most activation occurring during the first Ca^{2+} transient which tends to be larger than subsequent responses (Halet *et al.,* 2004). There are some indications that PKC does undergo incremental translocation during the series of rapid (~30s) Ca^{2+} oscillations that occur on top of the initial Ca^{2+} transient at fertilization (Halet *et al.,* 2004). However, there is no incremental translocation in response to the low frequency Ca^{2+} oscillations that occur over several hours. As with CaMKII, the PKC activity appears to track the overall pattern of Ca^{2+} changes and there is no obvious 'decoding' of the frequency of Ca^{2+} oscillations at this level. Again it is assumed that some substrates of PKC could act to integrate the pulses in PKC activity.

A different kind of target for Ca^{2+} oscillations at fertilization are the mitochondria. Mammalian eggs typically contain >100,000 mitochondria and rely on oxidative phosphorylation for ATP production throughout fertilization and the early cleavage divisions (Dumollard *et al.,* 2004). During the Ca^{2+} oscillations in fertilizing mouse eggs it has been shown that there are a series of oscillatory increases in reduction of FAD and NAD (Dumollard *et al.,* 2004b). It appears that Ca^{2+} increases in the cytosol result in Ca^{2+} increases in the mitochondrial matrix and this leads to stimulation of mitochondrial dehydrogenases. The direct stimulation of the mitochondria reduction could also explain the finding that the sperm-induced Ca^{2+} oscillations lead to a distinct and transient increase in ATP levels as monitored by firefly luciferase luminescence (Campbell and Swann, 2006).

Mitochondria have also been proposed to act as device for decoding Ca^{2+} oscillations in cells where the output is measured in terms of reduction mitochondrial NAD (Hajnoczky *et al.,* 1995). There are indication that mitochondria in eggs can act as a unit to decode Ca^{2+} oscillations into an analogue response. Ca^{2+} oscillations are probably causing Ca^{2+} pulses in the eggs mitochondria (Dumollard *et al.,* 2007). The Ca^{2+} stimulated NADH/FADH reduction that follows is also oscillatory but there is clearly some degree of memory and integration in that each increase in FADH, for example, outlasts each Ca^{2+} transient (Dumollard *et al.,* 2004b; Dumollard *et al.,* 2006). By the time we look at the ATP in the cytosol we find that these Ca^{2+} dependent events have lost their oscillatory pattern and instead there is a steady increase in ATP that is maintained for the duration of oscillations (Campbell and Swann, 2006). This is a rare example where we can trace the dynamics of a form of digital to analogue conversion in the egg's biochemistry. Unfortunately it is not known if a Ca^{2+} stimulated ATP increase has a direct function. It may be more significant that the ATP is not allowed to fall during the increased energy demands associated with Ca^{2+} pumping during Ca^{2+} oscillations. A transient fall in ATP levels in mouse oocytes has been shown to cause a decrease in the rate developmental to the expanded blastocyst stage after fertilization (Van Blerkom *et al.,* 1995).

Why does Ca^{2+} oscillate in an egg?

One question that often arises with regards to the Ca^{2+} signals in mammalian eggs is why there are oscillations at all. It has been known for some time that Ca^{2+} ionophore, or ethanol can activate mammalian eggs by causing a single large rise in Ca^{2+} (Colonna *et al.,* 1989; Ozil and Swann, 1995). Furthermore, so much of the Ca^{2+} signal seems to be transduced via CaMKII and yet egg activation and development can be achieved with a CA-CaMKII, which is presumably constant in activity. So oscillations in CaMKII are not necessary for egg activation and the question remains as to why Ca^{2+} levels normally oscillate?

One possibility is that the oscillations are simply a consequence of the non linear feedback loop of Ca^{2+} activated $InsP_3$ production that is intrinsic to PLCζ. This may well be the case, but it should be noted that many eggs such as those of sea urchins or frogs have many of the same signalling components and yet display a single Ca^{2+} increase at fertilization (Stricker 1999). The difference is not likely to be the absence or presence of PLCζ in mammalian eggs because even injection of the sperm factor into a frog egg (Wu *et al.,* 2001) causes only a single large Ca^{2+} increase. So the Ca^{2+} release machinery, or toolkit, can be put together in a way that does not lead to oscillations.

One reason why a single Ca^{2+} increase of the type seen in frog or sea urchins eggs is not seen in mammalian fertilization probably has to do with efficiency. A single Ca^{2+} increase generated by Ca^{2+} ionophore, or by electroporation to keep Ca^{2+} high for 5-10 minutes is generally not very efficient at activating eggs. Agents that cause such a single Ca^{2+} increase work best with mammalian eggs that are aged *in vivo*. When eggs are treated with a single large Ca^{2+} increase the activation rate of freshly ovulated mouse eggs is very low (Ozil and Swann, 1995; Toth *et al.,* 2006). A much better protocol for activation is to expose eggs to a single large Ca^{2+} increase and then follow this with a series

of extra smaller pulse of Ca^{2+}, that in effect resemble the pattern seen at fertilization (Toth *et al.,* 2006). A single 5-10 minute Ca^{2+} increase is often a poor stimulus for activation because, despite cyclin B degradation and polar body emission after one Ca^{2+} transient, the continued synthesis of cyclin B can lead to a return of cyclinB/CDK1 activity and the re-establishment of meiotic arrest in some eggs (Ducibella *et al.,* 2002). This is why the most general protocol for egg activation uses a Ca^{2+} ionophore plus either a protein synthesis inhibitor, or a protein kinase inhibitor, which block either the re-synthesis of cyclin B or activity of CDK1.

Whilst it is clear that a relatively short singular Ca^{2+} stimulus (5-10mins) is not always effective in activating eggs, a more difficult question is raised when a longer duration, but sustained, Ca^{2+} rise is applied to eggs. For example, electroporation can be used to cause prolonged elevations of Ca^{2+} in the egg and if the period of a Ca^{2+} increase is extended to more than 20 minutes, the egg activation can be effectively triggered (Ozil *et al.,* 2005). In fact it has been suggested that it is the total sum of elevated Ca^{2+} this is important in stimulating egg activation rather than any particular pattern of Ca^{2+} pulses (Ozil *et al.,* 2005; Toth *et al.,* 2006). This suggests that stimulating an egg to go through the first cell cycle is less dependent upon the form of Ca^{2+} increase so long as enough Ca^{2+} is released.

The answer to this second issue may be more subtle and connected with later events in development. The eggs that are activated by a single Ca^{2+} increase do not develop as well after implantation as ones activated by multiple Ca^{2+} pulses (Ozil *et al.,* 2005). This is consistent with studies that have shown that the pattern of Ca^{2+} oscillations during activation influences the size and morphology of post-implantation parthenogenetic rabbit embryos, or fertilized mouse embryos (Ozil and Huneau, 2001; Ozil *et al.,* 2006). In a similar vein the protocol of exposing mouse eggs to different duration Sr^{2+} treatments, to induced different durations of Ca^{2+} oscillations, has been shown to effect the cell composition of blastocysts (Bos-Mikich *et al.,* 1997). These data all imply that there might be some range of patterns of Ca^{2+} oscillations that is best for embryo development in mammals and that this is not connected with the immediate task of getting the embryo through the first cell cycle. This idea is supported by the development of eggs that are activated without any Ca^{2+} increase at all. Inhibitors of protein synthesis such as cycloheximide, or inhibitors of CDK1, such as roscovtine, can activate mouse eggs and stimulate them to proceed through the first couple of cell cycles without causing any Ca^{2+} increase (Rogers *et al.,* 2006). However, embryo development to the blastocyst stage is very poor compared to embryos activated by Sr^{2+} that causes Ca^{2+} oscillations (Rogers *et al.,* 2006). It is far from clear how the presence or absence of different patterns of Ca^{2+} oscillations can influence later development in mouse. It has been shown that either the absence or presence of different patterns of Ca^{2+} oscillations can influence the pattern of gene expression in mouse embryos (Rogers *et al.,* 2006; Ozil *et al.,* 2006). This serves to repose the question in terms of how Ca^{2+} changes during activation can influence later gene expression. Further progress on this and related questions will require that more of the downstream effects of Ca^{2+} are monitored with the same temporal resolution that we can apply to Ca^{2+} itself.

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